Pharmacology, Biochemistry and Behavior 134 (2015) 49–56

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

Involvement of nuclear factor kappa B in the maintenance of persistent inflammatory hypernociception

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ARTICLE INFO

Article history:
Received 18 August 2014
Received in revised form 7 April 2015
Accepted 15 April 2015
Available online 19 April 2015

Keywords:
Primary sensory neuron
Dorsal root ganglion
Chronic inflammatory pain
PKA
PKC
Nuclear factor kappa B

ABSTRACT

The pathophysiology of chronic inflammatory pain remains poorly understood. In this context, we developed an experimental model in which successive daily injection of prostaglandin E_2 (PGE_2) for 14 days into rat hind paws produces a persistent state of hypernociception (i.e. decrease in mechanical nociceptive threshold). This state persists for more than 30 days after discontinuing PGE_2 injection. In the present study, we investigated the participation of nuclear factor kappa B (NF-κB), in the maintenance of this process. Mechanical hypernociception was evaluated using the electronic von Frey test. Activation of NF-κB signaling was measured through the determination of NF-κB p65 subunit translocation to the nucleus of dorsal root ganglion neurons (DRG) by immunofluorescence and western blotting. Herein, we detected an increase in NF-κB p65 subunit translocation to the nucleus of DRG neurons along with persistent inflammatory hypernociception compared with controls. Intrathecal treatment with either dexamethasone or PDTC (NF-κB activation inhibitor) after ending of the induction phase of the persistent inflammatory hypernociception, curtailed the hypernociception period as well as reducing NF-κB p65 subunit translocation. Treatment with antisense oligonucleotides against the NF-κB p65 subunit for 5 consecutive days also reduced persistent inflammatory hypernociception. Inhibition of PKA and PKCε reduced persistent inflammatory hypernociception, which was associated with inhibition of NF-κB p65 subunit translocation. Together these results suggest that peripheral activation of NF-κB by PKA and PKC in primary sensory neurons plays an important role in maintaining persistent inflammatory pain.

1. Introduction

Chronic pain is frequently associated with functional alterations in the central and peripheral nervous systems, including an increase in primary sensitive fibers excitability and drastic changes in cellular phenotype with expression of additional neurotransmitters, receptors and enzymes (Liu and Wood, 2011). Concerning chronic pain due to inflammation, its underlying pathophysiology remains poorly understood, especially because its genesis involves mechanisms that are different from those that trigger acute inflammatory pain. One impediment is that there are few experimental models that mimic chronic inflammatory pain states in humans. Although some models, such as Complete Freund's Adjuvant (CFA)-induced inflammation, could be maintained for long periods, they mimic unresolved acute inflammation. The clinically important chronic pain cases are those in which the inflammatory process has been already resolved, but the pain persists. Therefore, a suitable model is essential to study the mechanisms underlying chronic inflammatory pain, which is a requisite for evaluating potentially effective novel therapies.

In an attempt to elucidate the mechanisms of chronic inflammatory pain, we developed an experimental model. This state is achieved in rats or mice by successive daily injection of prostaglandin E_2 (PGE_2) for 14 days, which produced a persistent inflammatory hypernociception (i.e. decrease in mechanical nociceptive threshold) lasting more than 30 days after discontinuing PGE_2 injections (Ferreira et al., 1990). Our

Abbreviations: ODNs, antisense oligonucleotides; COX, cyclooxygenase; DRG, dorsal root ganglion; CFA, Complete Freund's Adjuvant; GR, glucocorticoid receptor; IL-1β, interleukin 1 beta; IL-8, interleukin 8; NF-κB, nuclear factor kappa B; PIH, persistent inflammatory hypernociception; PDTC, pyrrolidine dithiocarbamate; PGE_2, prostaglandin E2; PKA, protein kinase A; PKC, protein kinase C.

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http://dx.doi.org/10.1016/j.pbb.2015.04.005
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results suggest that persistent inflammatory hypernociception is generated by frequent periods of sensitization of the primary nociceptive fibers and has duration of several weeks. It is important to note that the persistent inflammatory hypernociception state is blocked by analgesics of peripheral action, such as dipyrone and N-methyl morphine, but it is easily restored by small doses of PGE_2 or dopamine, indicating the contribution by peripheral mechanisms to this phenomenon (Ferreira et al., 1990).

The molecular events associated with the maintenance of persistent inflammatory hypernociception are not completely understood. A critical step in the development and maintenance of chronic pain states is the phenotypic change in primary nociceptive neurons probably occurring through gene modulation. In this context, it is possible that transcription factors, such as nuclear transcription factor kappa B (NF-κB) might play a role in rendering neuronal phenotypic changes. The NF-κB/Rel family has five members: p50, p52, p65/RelA, RelB, and c-Rel. NF-κB is mainly composed of p65/p50 heterodimers and homodimers of p50 or p65. In its normal state, NF-κB exists as a cytoplasmic complex with its inhibitory protein IκBα. During its activation, IκBα is phosphorylated and subsequently degraded, allowing NF-κB translocation into the nucleus, where it modulates the transcription of target genes (Li and Verma, 2002). The increasing interest in characterizing NF-κB involvement in mediating inflammatory pain stems from the large number of genes and cellular processes that it regulates. This transcription factor contributes to controlling developmental processes, neuronal plasticity, synaptic transmission, death and cellular defense. Some of the genes whose expression NF-κB modulates are nitric oxide synthase, cyclooxygenase (COX), cytokines, adhesion molecules, acute-phase proteins and dinorphine (Barnes and Adcock, 1997; O'Neill and Kaltschmidt, 1997). Regarding inflammatory pain, it was shown that intrathecal treatment with NF-κB inhibitors reduces thermal and mechanical hyperalgesia after peripheral inflammation induced by CFA in rats (Lee et al., 2004). Moreover, mice lacking the NF-κB p50 subunit present with reduced nociception in both phases of the formalin test, and reduced thermal hypernociception accompanied by reduced COX-2 expression in the spinal cord (Niederberger et al., 2007).

Taking into account these evidences, in the present study, we investigated whether activation of NF-κB is involved in the maintenance of persistent inflammatory hypernociception. As maintenance of persistent inflammatory hypernociception depends on a continuous activation of PKA and PKCε, we determined here if inhibition of these kinases has corresponding inhibitory effects on both NF-κB activation and this process (Villarreal et al., 2009b).

2. Material and methods

2.1. Animals

Male Wistar rats (180–220 g) were housed in temperature-controlled rooms (22–25 °C), with access to water and food ad libitum. All experiments were conducted in accordance with the International Association for the Study of Pain guidelines (Zimmermann, 1983) and with the approval of the Ethics Committee of the Faculty of Medicine of Ribeirao Preto (University of Sao Paulo). The animals were used only in a single experimental group.

2.2. Drugs

The agents used in this study were PGE_2, dexamethasone, H89 and PDTC (Sigma; St Louis, MO, USA). $PKC\varepsilon V_{1-2}$ peptide (a selective PKCε inhibitor) was obtained from SynPep Corp (Dublin, CA, USA). Stock PGE_2 solutions were prepared by dissolving the drug in ethanol (Merck) followed by dilution with 0.9% saline to decrease the ethanol concentration to 1%. Dexamethasone and PDTC were dissolved in 0.9% saline. PGE_2 was administered in a volume of 100 μL with a 27-gauge hypodermic needle, which was subcutaneously introduced near the third digit, with the needle tip reaching the middle of the plantar area.

2.3. Evaluation of mechanical nociceptive threshold

The mechanical nociceptive threshold was measured with an electronic pressure-meter. The rats were placed in acrylic cages ($12 \times 20 \times 17$ cm in height) on a wire grid floor, 15 to 20 min before beginning tests. After this adaptation period, the paws were poked 2 to 3 times. Before paw stimulation, the animals were docile quiet, without any exploratory or toilet movements and not resting over their paws. In these experiments, a pressure-meter, which consisted of a hand-held force transducer adapted with a 0.7-mm^2 polypropylene tip (electronic von Frey anesthesiometer; IITC Inc. Life Science Instruments, Woodland Hills, CA) was used. The investigator was trained to apply the polypropylene tip perpendicularly in between the five distal footpads with a gradual increase in pressure. A tilted mirror below the grid provided a clear view of the animal hindpaw. The test consisted of poking the hindpaw to provoke a flexion reflex followed by a clear flinch response after the paw withdrawal. The electronic pressure-meter automatically recorded the stimulus intensity when the paw was withdrawn. The maximal value of calibration range in which the pressure was linearly detectable by the equipment was 80 g. The animals were tested before and after treatments, and the results are expressed as the delta reaction force (grams) that was calculated by subtracting the value of the measurements of the right paw (treated paw) from that of the left paw (control naïve paw). Before starting the experiments, it was confirmed that there were no differences in the basal responses of the right and left paws (Vivancos et al., 2004).

2.4. Induction of persistent inflammatory hypernociception

Before starting the experiments, the basal responses of rats were recorded in the left and right hind paws. There are two phases of persistent inflammatory hypernociception. In the first phase, rats received daily intraplantar (i.pl.) injections of PGE_2 (100 ng/100 μL) for 14 consecutive days (i.e. induction period). This dose of PGE_2 was selected in previous studies in our laboratory (Ferreira et al., 1990; Sachs et al., 2002). The control group received vehicle (saline 100 μL) during the same period. Mechanical inflammatory hypernociception was evaluated daily before (0 h) and after (3 h) a PGE_2 injection with the electronic pressure meter test as described above. After the end of the induction period, the maintenance phase starts in which it is not necessary to administer additional inflammatory agents such as PGE_2 to maintain persistent inflammatory hypernociception for 30 days. Mechanical hypernociception was also evaluated during the maintenance phase at indicated time points depending on the treatment protocol.

2.5. Oligodeoxynucleotide p65 NF-κB subunit targeting

Antisense oligonucleotides (ODNs) were used to induce p65 NF-κB subunit knock-down in rat DRG neurons. The ODN sequence design was based on a previous study (Kitaoka et al., 2004): antisense sequence (5′-AAA CAG ATC GTA CAT GGC-3′); mismatch sequence (5′-GCC ATG GAC GAT CTG TTT-3′) were derived from the respective antisense sequences by scrambling eight bases. Rats received an intrathecal injection of either antisense, once a day (20 μg/day/10 μL saline) or mismatch (20 μg/day/10 μL saline) for 5 days. The treatment started at day 1 after the induction of persistent inflammatory hypernociception. Five days after ODN treatments, the L4–L6 DRGs on the ipsilateral side where PGE_2 was injected was removed to assess antisense ODN treatment efficacy.

2.6. Real-time PCR

p65 NF-κB subunit mRNA expression levels were evaluated in rat DRG receiving antisense and mismatch treatments using real-time PCR. Briefly, rats were euthanized by decapitation under anesthesia and the DRGs (3 from each rat; L4, L5 and L6) were harvested, homogenized in and left in contact with 1 mL of TRIzol reagent (for 10 min) and total RNA was extracted. The purity of total RNA was measured with a spectrophotometer and the wavelength absorption ratio (260/280 nm) was between 1.8 and 2.0 for all preparations. Reverse transcription of total RNA to cDNA was carried out with reverse transcription reaction (Superscript II, Gibco Life Technologies, Grand Island, NY, USA). Real-time PCR quantitative mRNA analysis was performed in an ABI Prism 7500 Sequence Detection System using the SYBR-green fluorescence system (Applied Biosystems, Warrington, UK) for quantification of amplicons. RT-PCR was performed in a 20 μL reaction volume and carried out with heating at 95 °C (10 min), and then 40 cycles of 94 °C (1 min), 56 °C (1 min) and 72 °C (2 min). Melting curve analysis was performed (65–95 °C) in order to verify that only one product was amplified. Samples with more than one peak were excluded. The data were analyzed with the comparative cycle threshold (CT) method. The expression of β-actin mRNA was used as an internal control in all samples. The primers used were as follows: p65 NF-κB subunit (sense, 5′-CGCTTTATCAAGGTCCTTACTC-3′; antisense, 5′-GCGTTTTGAGATAT TTGAAGGT-3′) and GAPDH (sense, 5′CTGAGTATGTCGTGGAGTCTA-3′; antisense, 5′CTGCTTCACCACCTTCTTGAT-3′).

2.7. Intrathecal injections

Intrathecal (i.t.) ODN injections were performed under isoflurane anesthesia (2%) (Ferreira and Lorenzetti, 1996). The dorsal fur of each rat was shaved, the spinal column was arched, and a 26-gauge needle was directly inserted into the subarachnoid space, between the L4 and L5 vertebrae. Correct i.t. positioning of the needle tip was confirmed by manifestation of a characteristic tail flick response. A 10 μL volume containing the test agent was slowly injected with a 100 μL Hamilton microsyringe. The animals regained consciousness approximately 1 min after discontinuing the anesthetic.

2.8. DRG immunofluorescence

An immunofluorescence assay was used to identify a semi-quantitative translocation of p65 NF-κB subunit to the nucleus of rat DRG neurons during the maintenance of persistent inflammatory hypernociception. All groups of rats were deeply anesthetized with urethane and perfused through the ascending aorta with saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (4 °C). After the perfusion, L5 DRG was dissected out, post fixed in the same fixative for 2 h, which was then, replaced overnight with 30% sucrose. All of the DRGs were embedded in OCT, and DRG sections (14 μm) were cut in a cryostat and processed for immunofluorescence. All of the sections were blocked with 2% BSA in 0.3% Triton X-100 for 1 h at room temperature and then incubated for 2 h at 4 °C with a polyclonal rabbit p65 NF-κB subunit (1:400, Santa Cruz, CA, USA). After washing with PBS, sections were incubated with AlexaFluo-594 conjugated secondary antibodies (Molecular Probes, Carlsbad, CA, USA) for 1 h at room temperature. Subsequently, sections were washed with PBS, and incubated with DAPI (1:3000) for nuclear staining. Finally, they were coverslipped with fluoromount and sealed with enamel. The absence of cross-reactivity of the secondary antibodies was verified by omitting primary antibody during incubation. Double-labeled sections were analyzed by confocal laser microscope (TCS SP5, Leica Leisertechnik, Heidelberg, Germany). Color images from double-labeling experiments were adjusted for contrast and brightness using Adobe Photoshop 9.0 software (Adobe, San Jose, CA).

2.9. Western blotting analysis

After ODN p65 NF-κB treatment, rats were decapitated and the dorsal root ganglia (DRG L4-6) were removed surgically. DRGs were homogenized in a lysis buffer containing protease inhibitors (Sigma; St Louis, MO, USA). The protein concentrations of the lysate were determined using the Bradford method. The protein samples were separated on an SDS/PAGE gel and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech) followed by the incubation with primary antibody against rabbit anti-p65 NF-κB antibody (sc-372, Santa Cruz Biotechnology) in filtered TBS-T buffer containing 5% milk powder overnight at 2–8 °C. Membranes were then incubated for 1 h at room temperature with an HRP-conjugated secondary antibody (1:5000; Jackson ImmunoResearch). The blots were visualized in an ECL solution (Amersham Pharmacia Biotech, Little Chalfont, UK) and exposed in a ChemiDoc MP Imaging System (Bio-Rad Laboratories, Hercules CA, USA).

2.10. Nuclear extract obtainment and western blotting

After induction of persistent inflammatory hypernociception, rats were treated with PDTC (30 ng/day once a day for 4 days), H-89 (H89, 27 μg/50 μL/paw unique injection) PKCε inhibitor (3 μg/50 μL/paw unique injection) or vehicle. Twenty-four hours after the treatments, rats were decapitated and the dorsal root ganglia (DRG L4–6) were removed surgically. DRGs were initially homogenized in a tube containing 180 μL of a buffer containing Tris 10 mM, NaCl 10 mM, $MgCl_2$ 3 mM, DTT 500 μM, EGTA 100 μM and protease inhibitor cocktail (Sigma, St. Louis, MO, USA) in ultrapure water, pH adjusted to 7.4. Extracts were briefly centrifuged at 1000 *g*, for 30 s and supernatants were collected. To cytoplasm lyses, 20 μL of triton X-100 5% was added in the supernatants and vortexed by 30 s. This solution was maintained in ice for 5 min. Resultant extracts were centrifuged at 6000 *g* for 6 min. Pellets formed were washed twice with buffer containing Tris 10 mM, NaCl 10 mM, $MgCl_2$ 3 mM, DTT 500 μM, EGTA 100 μM and protease inhibitor cocktail, and lysed with 50 μL of Rippa buffer (Sigma, St. Louis, MO, USA) containing protease inhibitor cocktail. These samples were vortexed for 2 min and centrifuged at 16000 *g* for 10 min. The resulting supernatant was taken as nuclear extract.

Samples of nuclear extracts had their protein content quantified by the Bradford method. Proteins were separated using electrophoresis gel SDS-PAGE (10% of polyacrylamide) followed by transfer to a nitrocellulose membrane. Nitrocellulose membrane was incubated with rabbit anti-p65 NF-κB antibody (sc-372, Santa Cruz Biotechnology) 1:250 in filtered TBS-T buffer containing 5% milk powder overnight at 2–8 °C. Secondary antibody (anti-rabbit, IgG) conjugated with peroxidase (1:5000) diluted in TBS-T buffer containing 5% milk was incubated with the membrane by 1 h. The characterization of nuclear extracts was performed using mouse anti-nucleophosmin antibody (Sigma, St. Louis, MO, USA) 1:300 in milk 5%, followed by incubation with secondary anti-mouse conjugated with peroxidase 1:10,000. Mouse anti-actin (Sigma, St. Louis, MO, USA) antibody 1:10,000 in milk 5%, followed by secondary anti-mouse conjugated with peroxidase 1:10,000 was used as a negative control of nuclear protein purity, but no significant signal was observed (data not shown). The blots were visualized in an ECL solution (ECL western blotting Systems, GE Healthcare, Little Chalfont, BKM, UK) and exposed in a ChemiDoc XRS + System (Bio-Rad Laboratories, Hercules CA, USA).

2.11. Data analyses and statistics

All results were analyzed using Prisma 4.0 software and are presented as the means ± S.E.M. The experiments were repeated at least twice. The number of rats indicated in the legends is relative to one single experiment and it was used for statistical analyses. Rats were used once. Differences between groups were evaluated by analysis of variance

(one-way ANOVA) followed by Bonferroni's *t* test. Statistical differences were considered to be significant at $P < 0.05$.

3. Results

3.1. Induction of persistent inflammatory hypernociception

Fig. 1 diagrammatizes the induction (panel A) and maintenance (panel B) phases of the persistent inflammatory hypernociception induced by PGE_2. The nociceptive threshold was evaluated by testing on each of 14 consecutive days the effect of applying increasing pressure (electronic von Frey test) on hind paw withdrawal on rats repeatedly injected during time with either PGE_2 or vehicle. Mechanical hypernociception was evaluated before and 3 h after PGE_2 injection of during the induction phase of persistent inflammatory hypernociception. After this induction phase, the nociceptive threshold was evaluated once a day for up to 30 days, which is the maintenance phase. During the first 4 days of PGE_2 administration, hypernociception peaked at 3 h after injection, followed by restoration to the control level after 24 h (i.e. vehicle injected). From the sixth to the tenth days (induction phase), the hypernociception intensity evaluated before the PGE_2 injection was similar to that seen in the third hour after administration of PGE_2 (Fig. 1A). Even though after 10 days, persistent inflammatory hypernociception seems to be established, it was previously verified that 14 consecutive days of PGE_2 administration are required for inflammatory hypernociception to persist for up to 30 days (Ferreira et al., 1990). In fact, there was already significant mechanical hypernociception on the 15th day (first day of the maintenance phase; Fig. 1B) before PGE_2 injection compared with the saline injected group. It is noteworthy that the persistent inflammatory hypernociception was not attributable to possible traumatic inflammatory lesions by the repetitive injections, because the saline treated control animals did not develop mechanical hypernociception during this period (Fig. 1A and B).

3.2. Inhibition of NF-κB activation reduces the maintenance phase of persistent inflammatory hypernociception

In order to evaluate the participation of NF-κB in the maintenance of the persistent inflammatory hypernociception, rats were treated intrathecally with vehicle (saline), dexamethasone or PDTC starting one day after the end of the induction phase of persistent inflammatory hypernociception. All treatments were performed once a day for 4 consecutive days. These two different NF-κB inhibitors significantly reduced persistent inflammatory hypernociception compared with their control (Fig. 2A–B).

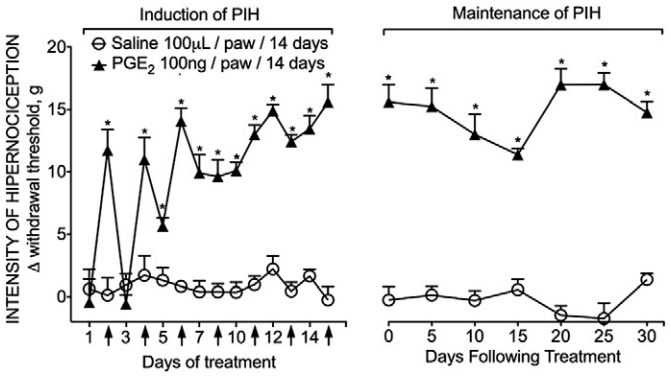

Fig. 1. Daily injection of PGE_2 induces persistent inflammatory hypernociception (PIH). Panel A shows the induction phase in which PGE_2 or saline is injected every day. The intensity of mechanical hypersensitivity was assessed twice each day before (0 h) and 3 h after intraplantar (i.pl.) injection of PGE_2 (100 ng/paw) or saline (100 μL/paw) treatments (indicated arrows). Panel B shows the persistent phase of hypernociception in which there is no injection of PGE_2. The mechanical hypernociception was evaluated with an electronic pressure meter test for rats. Data are expressed as the mean ± SEM of 5 rats per group. *$P < 0.05$ compared to saline control group.

In an attempt to further explore NF-κB involvement in the maintenance of persistent inflammatory hypernociception, ODN antisense was used to knockdown p65 NF-κB subunit expression, an ODN antisense directed against the p65 NF-κB subunit or an irrelevant mismatch were injected intrathecally once a day for 5 consecutive days starting one day after the end of induction of persistent inflammatory hypernociception. Corroborating with pharmacological treatments, ODN antisense also reduced persistent inflammatory hypernociception (Fig. 3A). Interestingly, persistent inflammatory hyperalgesia recovered after the interruption of ODN treatment (Fig. 3A). The effectiveness of ODN antisense treatment was confirmed by evaluating p65 subunit of NFκB mRNA and protein expression in the DRG (L4–6) (Fig. 3B and C).

After the evaluation of persistent inflammatory hypernociception, the group treated with vehicle or PDTC were used to evaluate NF-κB p65 subunit translocation to DRG neuron nuclei. In agreement with NF-κB involvement in the maintenance phase of persistent inflammatory hypernociception, p65 translocation from the cytoplasm to the nucleus increased as shown in representative western blotting and immunofluorescence images of rats that received 14 daily injections of PGE_2 compared to saline injected animals (Fig. 4A and B). Moreover, in agreement with the behavioral data shown in Fig. 2B, the PDTC treatment diminished NF-κB p65 subunit translocation to DRG neuron nuclei (Fig. 4A and B).

3.3. PKA and PKCε inhibition reduces persistent inflammatory hypernociception maintenance and NF-κB translocation

Our laboratory demonstrated that PKA and PKCε are activated during the maintenance phase of persistent inflammatory hypernociception (Villarreal et al., 2009b). Thus, we determined whether these kinases could be involved in NF-κB activation. Rats were locally (paw) treated with H-89 or PKCε inhibitor in the maintenance phase of persistent inflammatory hypernociception. Mechanical hypernociception was tested for 20 days using the electronic von Frey measurement. After the evaluation of hypernociception, DRG neurons (L4–6) were harvested and NF-κB p65 subunit translocation to the nuclei was determined.

As shown before, a single administration of H-89 (PKA inhibitor; Fig. 5A) or a PKCε inhibitor (Fig. 5B) reduced persistent inflammatory hypernociception (Villarreal et al., 2009b). Importantly, the inhibition of PKA or PKCε resulted in a reduction in NF-κB p65 subunit translocation to the nucleus of primary sensory neurons (Fig. 5C and D). These results suggest that PKA and PKCε activation during the maintenance phase results in NF-κB activation, which in turn is closely related to the maintenance of persistent inflammatory hypernociception.

4. Discussion

Chronic pain is a serious and debilitating problem that affects many people worldwide. In an attempt to investigate the mechanisms involved in genesis of chronic inflammatory pain, we developed in the 90s a model in which 14 daily injections of PGE_2 induces a state of persistent inflammatory hypernociception that is independent of further inflammatory stimuli. In the present study, we are showing that the activation of NF-κB in primary nociceptive neurons is critical to the maintenance of this type of persistent inflammatory hypernociception. Moreover, it seems that PKA and PKCε activation are responsible for triggering NF-κB activation.

One important characteristic of chronic pain is the fact that even after the resolution of the inflammatory process pain persists. Our experimental model used in the present study seems to mimic this state. In fact, even after stopping the administration of PGE_2, the hypernociceptive state is still observed. Besides PGE_2, other mediators such as dopamine, cytokines (e.g. TNF-α and IL-1β) and chemokines (e.g. IL-8) induce persistent inflammatory hypernociception (Sachs

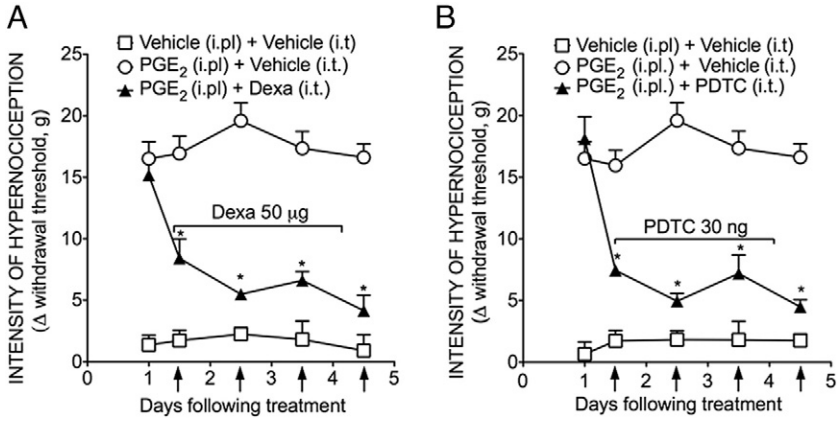

Fig. 2. NF-κB mediates the maintenance phase of persistent inflammatory hypernociception (PIH). Persistent inflammatory hypernociception was induced by daily injections of PGE_2 into rat paws for 14 days. After the induction phase, rats were treated for 4 days with dexamethasone (50 μg/10 μL/i.t. Panel A) or PDTC (30 ng/10 μL/i.t. Panel B). The mechanical hypernociception was evaluated daily 3 h after drugs treatment (indicated arrows) with an electronic pressure meter test for rats. Data are expressed as the mean ± SEM of 5 rats per group. $^*P < 0.05$ compared to PIH group.

et al., 2002). We assume that this experimental model indicates that repetitive inflammation for a period could be one of the underlying mechanisms for chronic pain and neuronal plasticity.

The main hypothesis raised to explain persistent inflammatory hypernociception is that phenotypic changes in the primary nociceptive neurons are responsible for the development of this process. These phenotypic alterations seem to be dependent on the synthesis of new proteins, since protein synthesis inhibition reduced persistent inflammatory hypernociception (Ferreira et al., 1990). Therefore, it is likely that transcriptional factors might play a role.

Among transcriptional factors, NF-κB is one of the most important and frequently involved in the production of local proinflammatory cytokines and chemoattractants by resident and migrating cells. This transcription factor also controls a large number of pro-inflammatory genes, including cytokines, cyclooxygenase-2, inducible nitric oxide synthase and others (Lee et al., 2009; Ndengele et al., 2008; O'Rielly and Loomis, 2008). In addition, several studies have shown that NF-κB is implicated in the pathogenesis of acute and chronic pain either inflammatory or neuropathic. Moreover, it has been shown that a blockade of NF-κB alters synaptic plasticity (Mattson and Camandola, 2001). Herein, we evaluated whether NF-κB participates in the maintenance of persistent inflammatory hypernociception. Firstly, we detected that pharmacologic inhibition of NF-κB with glucocorticoid or PDTC reduced the maintenance phase of persistent inflammatory hypernociception.

Supporting these behavioral and pharmacological data, the results also demonstrate that PDTC treatment reduced NF-κB activation in DRG neurons of rats during persistent inflammatory hypernociception. Considering that these drugs may have off target effects unrelated to NF-κB inhibition, we employed a genetic approach using ODN antisense to knockdown NF-κB p65 subunit expression. As intrathecal ODN antisense injection inhibited protein expression in peripheral sensory neurons, this approach is appropriate to selectively investigate the role of proteins expressed by primary nociceptive neurons (Khasar et al., 1996). Our results that persistent inflammatory hypernociception was reduced by ODN against p65 subunit, further support that NF-κB is an important transcription factor in the maintenance phase of persistent inflammatory hypernociception.

In accordance with our results, other investigators have shown that during inflammation and injury, respectively, the percentages of activated NF-κB immunoreactive neurons were significantly increased in dorsal root ganglia and the spinal cord of the rats (Bethea et al., 1998; Ma and Bisby, 1998). Corroborating these results, NF-κB p50 knockout mice have a reduced nociceptive response to acute and inflammatory noxious stimulation (Niederberger et al., 2007).

Nevertheless, most of these studies used models of ongoing inflammation, such as CFA-induced chronic inflammatory pain. In fact, our model of persistent inflammatory hyperalgesia, as far we identify, is the unique model in which pain hypersensitivity still present, at least, 30 days after discontinuing inflammatory stimulus injection. It is

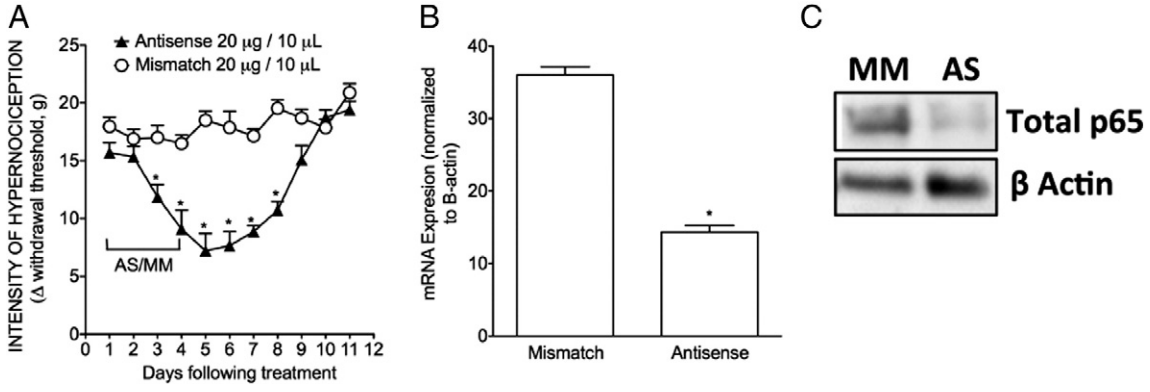

Fig. 3. The p65 NF-κB knockdown reduced persistent inflammatory hypernociception (PIH). PIH was induced by daily injections of PGE_2 into rat paws for 14 days. After the induction phase, rats were treated with ODN antisense or mismatch anti-NF-κB (20 μg/10 μL/i.t. Panel A). Mechanical hypernociception was evaluated before and 3 h after ODNs treatment. The NF-κB p65 subunit mRNA and protein expression in DRG was determined using qPCR and western blotting after treatment with ODN antisense anti-p65 NF-κB or mismatch (Panel B and C). Data are expressed as the mean ± SEM of 5 rats per group $^*P < 0.05$ compared to PIH group.

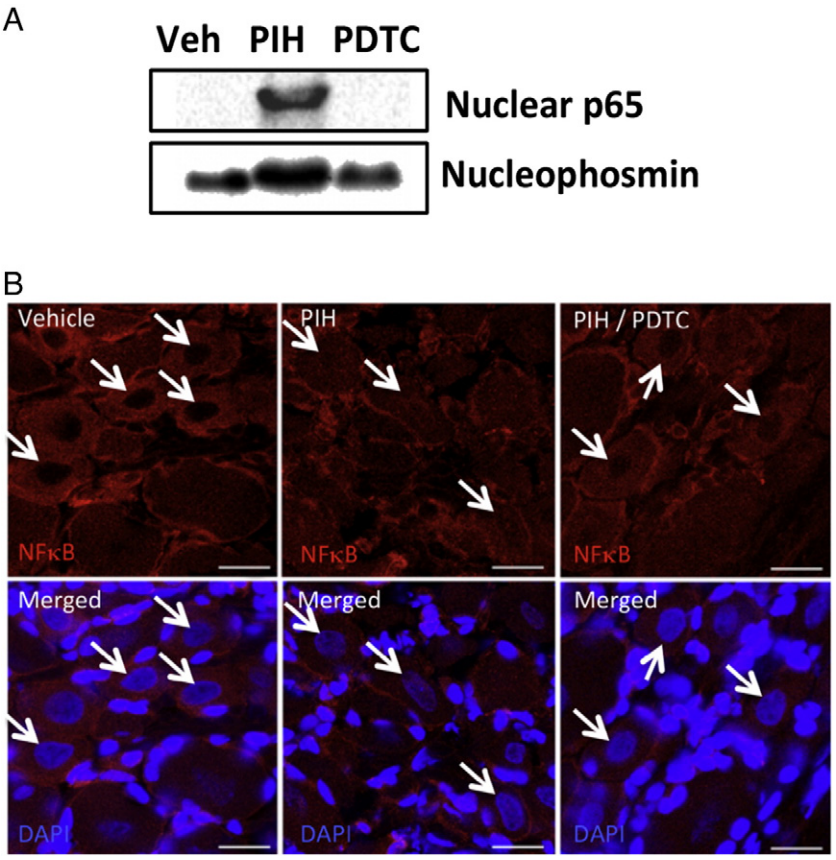

Fig. 4. Persistent inflammatory hypernociception (PIH) induces NF-κB activation. Nuclear p65 NF-κB expression in the DRG neurons of rats submitted to PIH was determined by western blotting (Panel A) and immunofluorescence (Panel B). One group of rats was treated with PDTC (30 ng/10 μL/i.t.) or vehicle treatment. DAPI staining was used as a nuclear marker (Panel B). Scale bars 20 μm.

noteworthy that persistent inflammatory hyperalgesia recovered after the interruption of treatment with ODN against p65 NF-κB. Although the exact explanation for this result is not clear, we could suggest that after the interruption of ODN treatment there is a turnover of p65 subunit which could be activated again by intracellular signaling pathways (eg. PKA and PKCε). Therefore, these delightful results indicate that even after peripheral (local of inflammation) stimulation has been ceased, NF-κB signaling in the dorsal root ganglions still activated and participate in the maintenance of persistent hyperalgesia.

The maintenance phase of persistent inflammatory hypernociception is dependent on PKA and PKCε, which result in increased expression of Nav1.8 channels (Villarreal et al., 2009b). Herein, we also verified that inhibition of PKA and PKCε activation reduces the maintenance phase of persistent inflammatory hypernociception, confirming previous a study with mice and rats (Sachs et al., 2009; Villarreal et al., 2009a). Importantly, PKA and PKCε inhibition also diminished the activation/translocation of NF-κB to the nucleus of DRG neurons. Therefore, it is likely that the 14 daily injections of PGE_2 induce a persistent inflammatory hypernociception that is maintained by PKA- and PKCε activation of NF-κB in DRG neurons. Since, PKA and PKCε activation is involved in the up-regulation of Nav1.8 expression during persistent inflammatory hypernociception (Villarreal et al., 2009a), we might speculate that this event is NF-κB signaling-dependent. It is important to mention that these results did not prove a direct action of PKA and PKCε on NF-κB. Nevertheless, there is evidence in the literature indicating that PKA and/or PKC can induce NF-κB-mediated effects. For instance, N-nitroso-N-methylurea and N-nitroso-N-ethylurea induce NF-κB activation via PKC in human keratinocytes (Moon, 2010), TNFα activates NF-κB in a PKA- and PKCδ-dependent manner (Takahashi et al., 2002), and PKA induces NF-κB-mediated Schwann cell differentiation into a myelinating phenotype (Yoon et al., 2008). Together these results can support our hypothesis that during persistent inflammatory hypernociception NF-κB activation is probably mediated by PKA and PKC.

One remarkable point warranting consideration is the locale in which NF-κB inhibitors are acting after intrathecal injection. We and others showed that besides acting in the spinal cord cells, substances, injected intrathecally, can also act in the DRGs (Ferrari et al., 2007; Gendron et al., 2006; Lorenzetti and Ferreira, 1996). Thus, in the present study, our hypothesis is that NF-κB inhibitors block translation of NF-κB-dependent genes by acting at the cell body level in the primary nociceptive neurons. Although, we evaluated the NF-κB p65 subunit translocation to the nucleus of primary sensory neurons in the DRGs, it is still necessary to consider the possibility of a NF-κB inhibitor spinal effect after intrathecal treatment. Indeed, such an agent could target glial cells in the spinal cord such as astrocytes and microglia cells, which are involved in chronic pain maintenance (Pan et al., 2010; Zhang et al., 2011). For instance, the NF-κB pathway in spinal glial cells has been implicated in neuropathic pain (Ledeboer et al., 2005; Meunier et al., 2007). Moreover, chronic constriction of the sciatic nerve induces microglia activation in the spinal cord (Colburn et al., 1999) which is dependent in part on NF-κB activation (Wilms et al., 2003) and probably is involved in nociceptive responses (Watkins et al., 1997). Even in the DRGs, NF-κB inhibitors could target satellite glial cells that seem to be participating in the genesis of chronic pain by producing proinflammatory mediators (Souza et al., 2013; Wang et al., 2011). Therefore, we cannot discard that NF-κB signaling activation in glial cells can account for the maintenance of persistent inflammatory hypernociception.

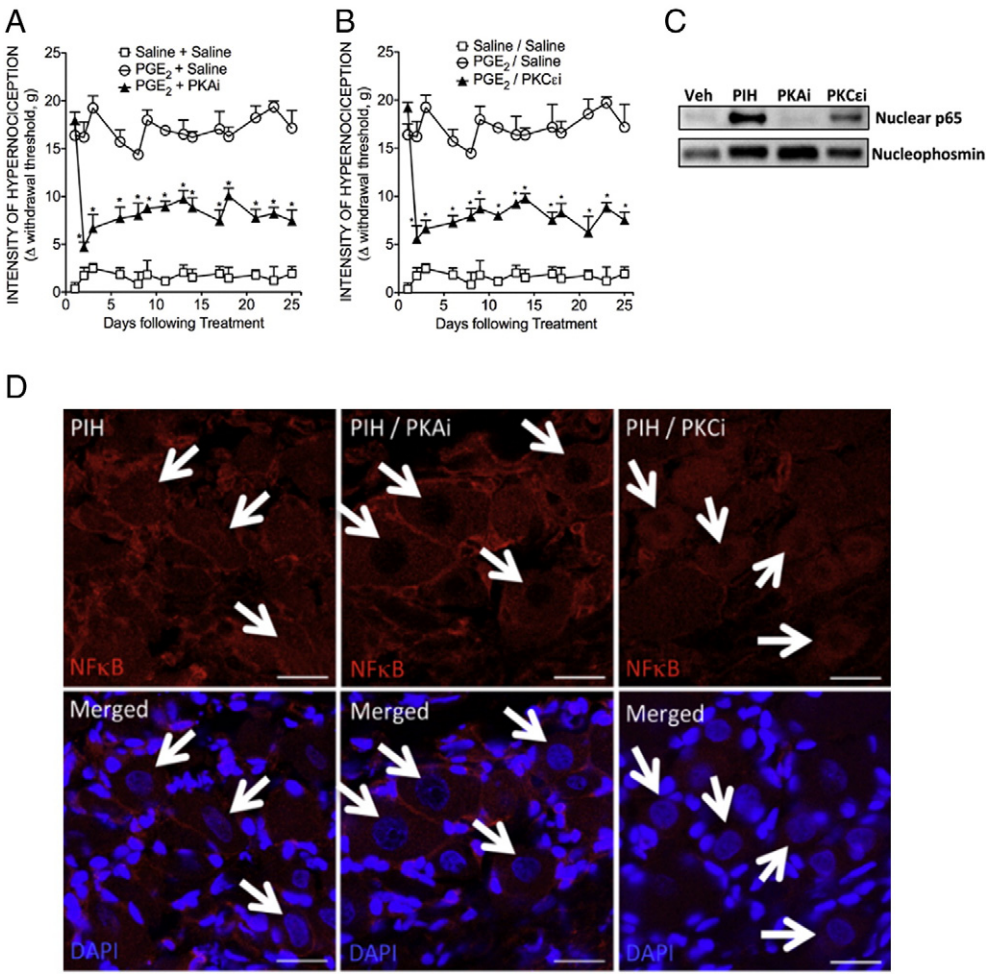

Fig. 5. PKA and PKCε mediate NF-κB activation during persistent inflammatory hypernociception (PIH). PIH was induced by daily injections of PGE_2 for 14 days in the rat paws. After the induction phase, rats were treated with a single dose of PKA inhibitor (H89, 27 μg/50 μL/paw) or PKCε inhibitor (3 μg/50 μL/paw). Mechanical hypernociception was evaluated before and 3 h after the treatments and once daily for 25 days (Panels A–B). Nuclear p65 NF-κB expression was determined in the DRGs by western blotting (Panel C) and immunofluorescence (Panel D). Data are expressed as the mean ± SEM of 5 rats per group. *$P < 0.05$ compared to PIH group. Scale bars: 20 μm.

In conclusion, the present study indicates that the activation of NF-κB signaling in primary sensory neurons is an important event involved in the maintenance of persistent inflammatory hypernociception induced by PGE_2 in rats. The results also indicate that PKA- and PKCε-dependent pathways might be triggering this signaling. More generally speaking, peripheral inhibition of NF-κB pathway signaling might be a suitable approach to control persistent inflammatory pain.

Acknowledgments

The authors gratefully acknowledge the technical assistance of Ieda R. dos Santos, Sergio R. Rosa, Eleni L. T. Gomes and Ana K. dos Santos and Dr. Peter S. Reinach for the language revision. The research leading to these results has received funding from São Paulo Research Foundation (FAPESP) under grant agreements no. 2011/19670-0 (Projeto Tematico) and 2013/08216-2 (Center for Research in Inflammatory Disease), from the University of São Paulo NAP-DIN under grant agreement no. 11.1.21625.01.0 and from the European Union Seventh Framework Programme [FP7-2007-2013] under grant agreement no. HEALTH-F4-2011-281608 (TIMER).

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